

**STABLE COMPOSITIONS FOR
MEASURING HUMAN NATRIURETIC PEPTIDES**

5 **Related Application Information**

This application is a continuation-in-part of U.S. Application No. 10/620,475, filed on July 16, 2003, which is hereby incorporated by reference.

Field of the Invention

10 The present invention relates to stable compositions, including, but not limited to, calibrators, controls and test samples, that can be used in ligand-binding assays, such as immunoassays, and methods for making said compositions.

Background of the Invention

15 Atrial natriuretic peptide (hereinafter referred to as "ANP"), brain natriuretic peptide (hereinafter referred to as "BNP"), C-type natriuretic peptide (hereinafter referred to as "CNP") and *Dendroaspis* natriuretic peptide (hereinafter referred to as "DNP") are each members of a family of hormones known as "natriuretic peptides". ANP and BNP share a wide spectrum of biological properties and belong to the cardiac natriuretic system. Both ANP and BNP are of 20 myocardial cell origin while CNP is of endothelial cell origin. DNP was isolated from the venom of the green mamba snake and possesses structural similarity to ANP, BNP and CNP.

25 BNP received its name because it was first isolated from porcine brain, thus "BNP" stood for "brain natriuretic peptide". However, because BNP belongs to the cardiac natriuretic system, "brain" has been changed to "B-type". Therefore, "BNP" now refers to "B-type natriuretic peptide".

30 ANP is secreted by the heart in the atria. BNP is secreted by the heart through the coronary sinus, predominantly from the cardiac ventricles. BNP is secreted as a 108 amino acid polypeptide precursor (See Valli et al., *J. Lab. Clin. Med.*, 134(5):437-444 (November 1999)). The mature form of BNP is made up of 32 amino acids with a 17 amino acid ring closed by a disulfide bond between two cysteine residues, an amino-terminal tail of 9 amino acids, and a carboxyl-terminal tail of 6 amino acids. ANP and CNP also have a 17 amino acid ring closed by

a disulfide bond between two cysteine residues. Eleven of the seventeen amino acids in the ring are conserved between the three molecules. In addition to the 17 amino acid ring structure, ANP has an amino-terminal tail of 6 amino acids and a carboxy-terminal tail of 5 amino acids. ANP is produced as a 126 amino acid pro-ANP form that is the major storage form of ANP. After 5 proteolytic cleavage between amino acids 98 and 99, the mature 28 amino acid peptide ANP is found in coronary sinus plasma (See Yandle, *J. Internal Med.*, 235:561-576 (1994)).

CNP is found in the brain and cerebral spinal fluid and is the most prevalent of the three peptides in the central nervous system. Little if any CNP is present in the heart. Pro-CNP is a 10 103 amino acid peptide that is processed into either CNP-53 (amino acids 51 to 103) or CNP-22 (amino acids 82 to 103) that are the active peptides. In addition the 17 amino acid ring structure, CNP-22 has an amino-terminal tail of 5 amino acids and contains no carboxy-terminal tail. CNP-53 is identical to CNP-22 except for a 31 amino acid extension at the amino terminal end.

15 As mentioned previously, DNP was isolated from the venom of the green mamba snake. The mature form of DNP is made up of 38 amino acids. DNP-like immunoreactivity (DNP-LI) has been reported in human plasma and the plasma concentration of DNP-LI has been found to be elevated in patients with congestive heart failure (See, Cataliotti, et al., *Mayo Clin. Proc.*, 76:111-1119 (2001)). Additionally, it is also known that the infusion of synthetic DNP results in 20 marked natriuresis and diuresis in association with increased plasma and urinary cyclic guanosine monophosphate. *Id.*

The measurement of BNP in human plasma in the general population has been found to reflect cardiac diseases, such as congestive heart failure, ischemic heart diseases, atrial 25 fibrillation and renal dysfunction. In fact, elevated levels of BNP in human plasma has been reported in heart disease, following acute myocardial infarction and during symptomless or subclinical ventricular dysfunction (See Mukoyama et al., *J. Clin. Invest.*, 87:11402-11412 (1991), Motwani et al., *Lancet*, 341:1109-1113 (1993), Yoshibayashi et al., *New Eng. J. Med.*, 327:434 (1992)). Increased circulating levels of ANP are seen in congestive heart failure, 30 chronic renal failure and in severe hypertension. The presence of CNP in human plasma remains

controversial with reports of its absence or presence as CNP-22 (See Yandle, *J. Internal Med.*, 235:561-576 (1994)).

A ligand binding assay is an analytical technique for measuring concentrations of substances commonly referred to as ligands that react selectively with specific binding proteins. Immunoassays that measure the concentrations of antigens that react selectively with specific antibodies are an example of a class of ligand binding assays.

Ligand binding assays, such as immunoassays, for measuring human natriuretic peptides in plasma are well-known in the art and are commercially available. These immunoassays require the use of at least one or two specific antibodies as well as at least one calibrator and, ideally, at least one control. Calibrators are used in ligand binding assays to calibrate instruments prior to calculating the sample result. The calibrators and controls used in such assays are typically made from human synthetic natriuretic peptides. Human synthetic natriuretic peptides are commercially available from a variety of sources. For example, human synthetic BNP is commercially available from Peptide Institute (Osaka, Japan), American Peptide Company, Inc. (Sunnyvale, California), Synpep Corporation (Dublin, CA) and Phoenix Pharmaceuticals, Inc. (Belmont, CA).

One of the problems with both natural and synthetic human natriuretic peptides is that they are unstable in plasma and serum. Specifically, enzymes, such as proteases, cleave these peptides. For example, proteases cleave BNP (natural and synthetic) at various locations along its amino acid chain. For example, protease cleavage is known to occur at the amino terminus of BNP between amino acids 2-3 (Shimizu et al., *Clinica Chimica Acta*, 316:129-135 (2002)) and at its carboxy terminus between amino acids 30-32. Moreover, endopeptidase cleavage of BNP is also known in the art (Davidson and Struthers, *J. Hypertension*, 12:329-336 (1994)). Such cleavage is problematic because in order for calibrators and controls to function properly in an assay, a human natriuretic peptide containing immunoreactive or specific ligand binding sites must be present at the intended concentration during the assay.

Thereupon, as a result of this instability, the calibrators and controls used in such assays are sold either in lyophilized form (such as in the Shionoria assay available from Shionogi & Co., Ltd., Osaka, Japan) or frozen (such as in the Triage® assay available from Biosite, Inc., San Diego, California). Calibrators and controls in lyophilized form must be reconstituted in a solvent prior to use in an assay. Once reconstituted, these calibrators and controls must be used within a specific time because they are very unstable. Calibrators and controls that are frozen (usually at temperatures of about -20°C or colder) remain frozen until thawed for use in the assay. These calibrators and controls are thawed at room temperature and then vortexed or inverted to make the calibrators and controls homogenous prior to testing. Frozen calibrators and controls cannot be re-frozen and can only be used once (i.e. are single-use) and then discarded.

In addition to the calibrators and controls, immunoassays require the use of at least one test sample. Test samples are normally biological samples derived from serum, plasma, whole blood or other bodily fluids (normally from a human patient). The levels of at least one human natriuretic peptide in the test sample is quantified in the immunoassay. Some samples, with extremely high levels of natriuretic peptides, must be diluted prior to analysis in order to get an accurate quantification of the peptide level. However, as described earlier, one of the problems with natural human natriuretic peptides, even in test samples, is that they are unstable.

Thereupon, as a result of this instability, after a test sample is obtained and once it is diluted, it must be used in an immunoassay within a short period of time (i.e., less than one hour, see ADVIA Centaur® Assay Manual 06300497, Rev. C, 2003-06). Such a short period of instability can be problematic for a laboratory conducting such the immunoassays, particularly if the volume of immunoassays to be conducted is large.

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Thereupon, there is a need in the art for new calibrators and controls for use in human natriuretic ligand binding assays that are stable for extended periods of time and are easy and quick to use and do not need to be reconstituted or thawed prior to use in such assays. Additionally, there is also a need in the art for test samples that can be used in such assays that are stable for extended periods of time.

Summary of the Invention

In one embodiment, the present invention relates to stable liquid calibrators and controls that can be used in ligand binding assays, such as immunoassays, for measuring the level of natriuretic peptides in a test sample. The stable liquid calibrators and controls of the present invention have a pH of from about 4.0 to about 6.5, preferably from about 5.0 to about 6.0.

Moreover, the calibrators and controls of the present invention comprise at least one natriuretic peptide, preferably at least one human synthetic natriuretic peptide, such as a human synthetic atrial natriuretic peptide, human synthetic B-type natriuretic peptide, human synthetic C-type natriuretic peptide or human synthetic *Dendroaspis* natriuretic peptide. In addition to the natriuretic peptide, the calibrators and controls can also comprise at least one buffer, or at least one acid, or at least one base, or combinations of at least one buffer, at least one acid and/or at least one base. Examples of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof. Examples of acids that can be used include, but are not limited to, acetic acid, citric acid, diethylenetriaminepentaacetic acid, hydrochloric acid or combinations thereof. Examples of bases that can be used include, but are not limited to, sodium hydroxide.

The calibrators and controls can also comprise at least one diluent. The diluent can comprise at least one natriuretic stabilizing compound and at least one biocide. Examples of natriuretic stabilizing compounds that can be used include, but are not limited to, at least one protein or polymer. Examples of proteins that can be used include, but are not limited to, bovine serum albumin, bovine gamma globulin, or non-fat dry milk. Examples of polymers that can be used include, but are not limited to, polyethylene glycol, dextran, dextran sulfate or polyvinyl pyrrolidone. The diluent can further contain at least one buffer, at least one acid, at least one base, or combinations thereof. Examples of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such

as mono-/di-sodium phosphate) or combinations thereof. Examples of acids that can be used include, but are not limited to, acetic acid, citric acid, diethylenetriaminepentaacetic acid, hydrochloric acid or combinations thereof. Examples of bases that can be used include, but are not limited to, sodium hydroxide.

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The calibrators and controls of the present invention remain stable for long periods of time under a variety of storage conditions. Specifically, the calibrators and controls of the present invention remain stable when stored for extended periods of time at a temperature of from about 2 to about 8°C. Additionally, the calibrators and controls of the present invention 10 remain stable when used at ambient temperatures and for limited periods of time (such as between 1 minute and 60 minutes, preferably for a period of about 20 to about 30 minutes) at temperatures of from about 30 to about 40°C, preferably at a temperature of about 34°C.

In a second embodiment, the present invention relates to a method of making stable 15 liquid calibrators and controls for use in a ligand binding assay. The first step of the method involves mixing at least one diluent with at least one human synthetic natriuretic peptide to form a liquid calibrator or control. The second step involves measuring the pH of the liquid calibrator or control. Depending upon the pH of the liquid calibrator or control measured in the first step, the pH of the liquid calibrator or control may be adjusted so that the pH is in the range of from 20 about 4.0 to about 6.5, preferably in the range from about 5.0 to about 6.0. The pH of the calibrator or control can be adjusted using at least one buffer, or at least one acid, or at least one base or combinations of at least one buffer, at least one acid and/or at least one base . Examples 25 of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof. Examples of acids that can be used include, but are not limited to, acetic acid, citric acid, diethylenetriaminepentaacetic acid, hydrochloric acid or combinations thereof. An example of a base that can be used is sodium hydroxide.

The human synthetic natriuretic peptide used in the above-described method can be human synthetic atrial natriuretic peptide, human synthetic B-type natriuretic peptide, human synthetic C-type natriuretic peptide or human synthetic *Dendroaspis* natriuretic peptide.

5 The diluent used in above described method can comprise at least one natriuretic stabilizing compound and at least one biocide. Examples of natriuretic stabilizing compounds, include, but are not limited to, at least one protein or polymer. Examples of proteins that can be used include, but are not limited to, bovine serum albumin, bovine gamma globulin or non-fat dry milk. Examples of polymers that can be used include, but are not limited to, polyethylene glycol, dextran, dextran sulfate or polyvinyl pyrrolidone. The diluent can further contain at least one buffer, at least one acid, at least one base, or combinations thereof. Examples of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof. Examples of acids that can be used include, but are not limited to, acetic acid, citric acid, diethylenetriaminepentaacetic acid, hydrochloric acid or combinations thereof. Examples of bases that can be used include, but are not limited to, sodium hydroxide.

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In a third embodiment, the present invention relates to a stable test sample that can be used in ligand binding assays, such as immunoassays, for measuring the level of at least one natriuretic peptide in said test sample. The stable test sample of the present invention has a pH of from about 4.0 to about 6.5, preferably from about 5.0 to about 6.0.

20 The test sample of the present invention comprises at least one biological sample that contains at least one natriuretic peptide, preferably, at least one human natural natriuretic peptide, such as a human natural atrial natriuretic peptide, human natural B-type natriuretic peptide, human natural C-type natriuretic peptide or human natural *Dendroaspis* natriuretic peptide. The biological sample used in the test sample is derived from human serum, human plasma, human whole blood or other human bodily fluids. The test sample can also comprise at least one diluent. The amount of diluent in the test sample is from about 5% to about 95% (v/v), preferably from about 20% to about 90% (v/v). The diluent can comprise at least one natriuretic

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stabilizing compound and, optionally, at least one biocide. Examples of natriuretic stabilizing compounds that can be used include, but are not limited to, at least one protein or polymer. Examples of proteins that can be used include, but are not limited to, bovine serum albumin, bovine gamma globulin, or non-fat dry milk. Examples of polymers that can be used include,
5 but are not limited to, polyethylene glycol, dextran, dextran sulfate or polyvinyl pyrrolidone. The diluent can further contain at least one buffer, or at least one acid, or at least one base, or combinations of at least one buffer, at least one acid and/or at least one base . Examples of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric
10 acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof. Examples of acids that can be used include, but are not limited to, acetic acid, citric acid, diethylenetriaminepentaacetic acid, hydrochloric acid or combinations thereof. Examples of bases that can be used include, but are not limited to, sodium hydroxide.

15 The test sample of the present invention remains stable for long periods of time under a variety of storage conditions. Specifically, the test sample of the present invention remain stable when used at ambient temperatures (for a period of at least about twenty-four (24) hours).

In a fourth embodiment, the present invention relates to a method of making a stable test
20 sample for use in a ligand binding assay. The method involves mixing from about 5% to about 95% (v/v), preferably from about 20% to about 90% (v/v), of at least one diluent with at least one a biological sample derived from serum, plasma, whole blood or other bodily fluids, to form a stable test sample having a pH of from about 4.0 to about 6.5, preferably in the range from about 5.0 to about 6.0. The biological sample contains at least one natriuretic peptide that can be
25 a human natriuretic peptide, such as, but not limited to, a human natural atrial natriuretic peptide, human natural B-type natriuretic peptide, human natural C-type natriuretic peptide or human natural *Dendroaspis* natriuretic peptide. The biological sample is derived from serum, plasma, whole blood or other bodily fluids.

30 The diluent used in above described method can comprise at least one natriuretic stabilizing compound and, optionally, at least one biocide. Examples of natriuretic stabilizing

compounds, include, but are not limited to, at least one protein or polymer. Examples of proteins that can be used include, but are not limited to, bovine serum albumin, bovine gamma globulin or non-fat dry milk. Examples of polymers that can be used include, but are not limited to, polyethylene glycol, dextran, dextran sulfate or polyvinyl pyrrolidone. The diluent can further

5 contain at least one buffer, or at least one acid, or at least one base, or combinations of at least one buffer, at least one acid and/or at least one base . Examples of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof.

10 Examples of acids that can be used include, but are not limited to, acetic acid, citric acid, diethylenetriaminepentaacetic acid, hydrochloric acid or combinations thereof. Examples of bases that can be used include, but are not limited to, sodium hydroxide.

In a fifth embodiment, the present invention relates to a method of stabilizing a test sample for use in a ligand binding assay. The method involves mixing from about 5% to about 15 95% (v/v), preferably from about 20% to about 90% (v/v), of at least one diluent with at least one a biological sample derived from serum, plasma, whole blood or other bodily fluids, to form a stable test sample having a pH of from about 4.0 to about 6.5, preferably in the range from about 5.0 to about 6.0. The biological sample contains at least one natriuretic peptide that can be

20 a human natriuretic peptide, such as, but not limited to, a human natural atrial natriuretic peptide, human natural B-type natriuretic peptide, human natural C-type natriuretic peptide or human natural *Dendroaspis* natriuretic peptide. The biological sample is derived from serum, plasma, whole blood or other bodily fluids.

25 The diluent used in above described method can comprise at least one natriuretic stabilizing compound and, optionally, at least one biocide. Examples of natriuretic stabilizing compounds, include, but are not limited to, at least one protein or polymer. Examples of proteins that can be used include, but are not limited to, bovine serum albumin, bovine gamma globulin or non-fat dry milk. Examples of polymers that can be used include, but are not limited to,

30 polyethylene glycol, dextran, dextran sulfate or polyvinyl pyrrolidone. The diluent can further contain at least one buffer, or at least one acid, or at least one base, or combinations of at least

one buffer, at least one acid and/or at least one base . Examples of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof.

5 Examples of acids that can be used include, but are not limited to, acetic acid, citric acid, diethylenetriaminepentaacetic acid, hydrochloric acid or combinations thereof. Examples of bases that can be used include, but are not limited to, sodium hydroxide.

The stabilized test sample prepared by the above-identified method can be used
10 immediately after preparation in a ligand binding assay, or can be used at a much later period in time in a ligand binding assay. For example, when the stabilized test sample is maintained in a laboratory conditions at ambient temperatures, it can be used in a ligand binding assay at least twenty-four (24) hours after being stabilized pursuant to the above-described method.

15 Brief Description of the Figures

Figure 1 shows graphs demonstrating the stability of calibrators having varying pH's at 37°C over time.

Figure 2 shows graphs demonstrating the stability of calibrators having varying pH's at
20 2-8°C over time.

Figure 3 shows graphs demonstrating the stability of calibrators having varying pH's at 37°C and 2-8°C over time.

25 Detailed Description of the Invention

In one embodiment, the present invention relates to stable calibrators and controls that can be used in ligand binding assays, such as immunoassays. More specifically, the present invention relates to stable calibrators and controls that are in liquid form and can be used in ligand binding assays to measure the levels of at least one natriuretic peptide in a test sample.

30 The test sample comprises at least one a biological sample that is derived from plasma, serum, whole blood or other bodily fluids, such as, but not limited to, saliva, sputum, etc., as long as the

bodily fluids are stable in their collection technique. The biological sample also contains at least one natriuretic peptide. Because the calibrators and controls of the present invention are in liquid form, they do not need to be reconstituted or defrosted prior to use like the lyophilized and frozen calibrators and controls known in the art. Additionally, the calibrators and controls of the 5 present invention are stable for long periods of time under a variety of storage conditions and temperatures.

The calibrators and controls of the present invention contain at least one natriuretic peptide and thus can be used in ligand binding assays to measure the level of these peptides in a 10 test sample. Either natural or synthetic natriuretic peptides can be used in the calibrators and controls of the present invention. Preferably, the natriuretic peptides are synthetic natriuretic peptides and are human, such as, human synthetic atrial natriuretic peptide, human synthetic B-type natriuretic peptide, human synthetic C-type natriuretic peptide or human synthetic 15 *Dendroaspis* natriuretic peptide. Human synthetic atrial natriuretic peptides, human synthetic B-type natriuretic peptides, human synthetic C-type natriuretic peptides and human synthetic *Dendroaspis* natriuretic peptides are commercially available from a variety of sources, including, but not limited to, Peptide Institute (Osaka, Japan), American Peptide Company, Inc. (Sunnyvale, CA), Synpep Corporation (Dublin, CA) and Phoenix Pharmaceuticals, Inc. (Belmont, CA). The amount of natriuretic peptides in the calibrators of the present invention is 20 from 0 to about 10,000 pg/mL, preferably in the amount of from about 25 to about 5,000 pg/mL. The amount of natriuretic peptides in the controls of the present invention is from about 25 to about 4000 pg/mL, preferably in the amount of from about 40 to about 2000 pg/mL.

In addition to the natriuretic peptides, the calibrators and controls are in a diluent that 25 comprises a number of components. Any diluent can be used in the calibrators and controls of the present invention. For example, a diluent that can be used in the calibrators and controls of the present invention can be made using techniques known in the art or can be purchased from a commercially available source. Combinations of both custom made diluents and commercially purchased diluents are also contemplated within the scope of the present invention.

The composition of the diluent can vary depending upon the calibrator or control. For example, the diluent can comprise water.

The diluent can also comprise at least one natriuretic stabilizing compound. As used herein, the term “natriuretic stabilizing compound” refers to a compound that can be used to stabilize a natriuretic peptide and prevent its degradation by enzymes, such as proteases. Several natriuretic stabilizing compounds are known in the art and can be used in the calibrators and controls of the present invention. These compounds include, but are not limited to, proteins, polymers and protease inhibitors. Examples of proteins that can be used include, but are not limited to, bovine serum albumin (BSA), bovine gamma globulin (bovine IgG), or non-fat dry milk (such as Nestlé® Carnation® Nonfat Dry Milk). Examples of polymers that can be used include, polyethylene glycol (having a molecular weight of from about 2,000 to about 20,000 daltons), dextran (having a molecular weight of from about 5,000 to about 670,000 daltons), dextran sulfate (having a molecular weight of about 5,000 daltons or about 1,000,000 daltons) or polyvinyl pyrrolidone (having a molecular weight of about 40,000 daltons). Bovine serum albumin, bovine IgG, polyethylene glycol, dextran, dextran sulfate and polyvinyl pyrrolidone can all be purchased from Sigma Aldrich, St. Louis, MO. Examples of protease inhibitors that can be used include, but are not limited to, EDTA or aprotinin. It is known in the art that EDTA at about 1mg/mL blood and aprotinin at approximately 50 kIU/mL of blood (Murdoch et al, *Heart*, 78:594-597 (1997)) can be used to help prevent BNP proteolysis. Protease inhibitors, such as EDTA and aprotinin can be purchased from Sigma Aldrich, St. Louis, MO. The amount of natriuretic stabilizing compound in the diluent can be from about 0.01 mg/mL to about 400 mg/mL, preferably in the amount of from about 1 mg/mL to about 50 mg/mL.

Additionally, the diluent can also comprise and at least one biocide and/or preservative. As used herein, the term “biocide” refers to a substance that can be used to kill a variety of different organisms. Suitable biocides and/or preservatives for use in the diluent can be determined using routine techniques by those skilled in the art. Examples of suitable biocides and/or preservatives that can be used in the present invention, include, but are not limited to ProClin® 300 (Sigma-Aldrich, St. Louis, MO) (The active ingredients of ProClin® 300 are 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) and sodium azide.

ProClin® 300 also contains a number of inert ingredients such as a modified glycol and alkyl carboxylate. The amount of biocide and/or preservative in the diluent can be from about 0.001 mg/mL to about 50 mg/mL, preferably in the amount of from about 0.1 mg/mL to about 10 mg/mL.

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Furthermore, the diluent can also comprise at least one buffer, or at least one acid, or at least one base or combinations of at least one buffer, at least one acid and/or at least one base . Examples of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof. It is preferred that the buffer have a good buffering capacity at the desired pH so that it can stabilize the pH of the diluent and the resulting calibrator or control. Examples of acids that can be used include, but are not limited to, acetic acid, diethylenetriaminepentaacetic acid (DTPA), hydrochloric acid (HCl) or combinations thereof.

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15 The amount of buffer or acid in the diluent can be from about 1 mM to about 500 mM, preferably in the amount of from about 5 to about 200 mM. An example of a base that can be used includes, but is not limited to, sodium hydroxide (NaOH). The amount of base in the diluent can be from about 0.01 mM to about 50 mM, preferably in the amount of from about 1.0 mM to about 10 mM.

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The diluent can also comprise sodium chloride (NaCl) and/or at least one detergent, such as Tween® (Any type of Tween® can be used, including, but not limited to, Tween® 20, Tween® 40, Tween® 60, which are commercially available from Sigma-Aldrich, St. Louis, MO). The amount of NaCl in the diluent can be from about 25 mM to about 500 mM, preferably in the amount of from about 100 mM to about 400 mM. The amount of detergent in the diluent can be from about 0.01 mg/mL to about 10 mg/mL, preferably in the amount of from about 0.1 mg/mL to about 3.0 mg/mL.

25 In addition, as mentioned above, commercially available diluents can be used. For example, the diluent known as “MEIA2” that is commercially available as IMx MEIA #2 Diluent Buffer (No.8374-04) from Abbott Laboratories, the assignee of the present invention,

can be used. MEIA2 contains 0.06 M Tris buffer (Sigma-Aldrich, St. Louis, MO), 0.3 M NaCl, 0.1% w/v NaN₃ and has a pH of 7.5.

In addition to the diluent, the calibrators and controls can also contain at least one buffer,
5 or at least one acid, or at least one base, or combinations of at least one buffer, at least one acid
and/or at least one base . These buffers, bases or acids can be present in the calibrators and
controls in addition to the buffers, bases and/or acids present in the diluent or as separate
components if the diluent does not contain any buffers, bases and/or acids. Examples of buffers
that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic
10 acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium
citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations
thereof. Examples of acids that can be used include, but are not limited to, acetic acid, citric
acid, diethylenetriaminepentaacetic acid (DTPA), hydrochloric acid (HCl) or combinations
thereof. Examples of bases that can be used include, but are not limited to, sodium hydroxide.
15 The amount of buffer, acid, base or combinations thereof in the calibrator or control can be from
about 1 mM to about 500 mM, preferably in the amount of from about 5 to about 200 mM.

As mentioned above, the calibrators and controls of the present invention are stable and
are in liquid form. The key to the stability of the calibrators and controls of the present invention
20 is pH. More specifically, it has been discovered that the liquid calibrators and controls of the
present invention are stable at a pH of from about 4.0 to about 6.5. Preferably, the pH of the
liquid calibrators and controls of the present invention is from about 5.0 to about 6.0.

The calibrators and controls of the present invention having a pH of from about 4.0 to
25 about 6.5 are stable for long periods of time and under a variety of storage conditions. For
example, the calibrators and controls of the present invention remain stable when stored at
temperatures of from about 2 to about 8°C for a period of about twelve (12) months or more.
The ability to store the calibrators and controls of the present invention at about 2°C to about 8°C
30 is very convenient and allows for multiple-use of these reagents. In contrast, the frozen
calibrators and controls known in the art are single-use reagents that have to be defrosted prior to
conducting an assay. These calibrators and controls cannot be refrozen and have to be thrown

away after being defrosted and used in an assay. Lyophilized controls known in the art, are only stable for 1 week at 2-8°C after reconstitution and must be discarded.

Additionally, the calibrators and controls of the present invention having a pH of from about 4.0 to about 6.5 remain stable for several hours when used under laboratory conditions at ambient temperatures. Additionally, the calibrators and controls of the present invention are stable for limited periods of time (such as, but not limited to, about 1 minute to about 60 minutes, preferably for a period of about 20 minutes to about 30 minutes) when exposed to temperatures of from about 30 to about 40°C, preferably at a temperature of about 34°C.

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In another embodiment, the present invention relates to methods for making stable calibrators and controls for use in ligand binding assays, such as immunoassays. The method of the present invention involves mixing at least one diluent with at least one natriuretic peptide to form a liquid calibrator or control. The diluent can have any of the compositions previously described herein and the natriuretic peptide can be any of the natriuretic peptides also previously described herein. Preferably, the diluent comprises at least one buffer having a good buffering capacity at the desired pH. Optionally, once the diluent has been prepared, it can optionally be heated to a temperature of from about 50 to about 60°C for a period of from about 30 minutes to about 24 hours. Preferably, the diluent is heated in a water bath at a temperature of about 55°C for a period of about 1 hour.

The diluent and natriuretic peptide are mixed together at a temperature of from about 15°C to about 30°C, preferably at a temperature of from about 19°C to about 23°C, until a homogenous solution is obtained. Once a homogenous liquid calibrator or control has been prepared, the pH is measured using a pH meter using techniques known in the art. If the pH of the liquid calibrator or control is determined to be in the range of from about 4.0 to about 6.5, then the pH of the liquid calibrator and control does not require any adjustment or stabilization and can be subjected to further processing steps such as those described in more detail below. However, if the pH of the liquid calibrator or control is not in the range of from about 4.0 to about 6.5, then the pH is adjusted or stabilized using routine techniques known in the art. For example, if the pH of the liquid calibrator or control is determined to be less than about 4.0, then

at least one base, such as sodium hydroxide, at least one buffer, or combinations thereof, is added to the liquid calibrator or control in small quantities, preferably drop wise, and the pH measured repeatedly until the pH of the liquid calibrator or control is in the range of from about 4.0 to about 6.5. Likewise, if the pH of the liquid calibrator or control is determined to be greater than

5 then at least one buffer, at least one acid or combinations thereof is added to the liquid calibrator or control in small quantities, preferably drop wise, and the pH measured repeatedly until the pH of the liquid calibrator or control is in the range of from about 4.0 to about 6.5.

Examples of buffers that can be used include, but are not limited to, acetate buffers (such as sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium

10 citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof. Examples of acids that can be used include, but are not limited to, acetic acid, citric acid, diethylenetriaminepentaacetic acid, hydrochloric acid or combinations thereof.

15 Once a liquid calibrator or control having a pH in the range of from about 4.0 to about 6.5 has been prepared, then the calibrator or control can optionally be heated to a temperature of from about 50° to about 60°C for a period of from about 30 minutes to about 24 hours.

Preferably, the calibrator or control is heated in a water bath at a temperature of about 55°C for a period of about 1 hour. After the calibrator or control has been heated, it can then be subjected

20 to further processing steps such as those described below.

Once a liquid calibrator or control having a pH in the range of from about 4.0 to about 6.5 has been prepared, then the calibrator or control can be subjected to further processing steps, such as, but not limited to, vortexing and/or filtering, using techniques known in the art. Once

25 these steps have been completed, then the calibrator or control is placed into bioclean or sterile vials (plastic or siliconized glass) and labeled accordingly. Once the calibrators or controls are placed into the bioclean or sterile vials, they can be stored for use in an assay at a temperature of from about 2°C to about 8°C for up to 12 months or more.

30 In third embodiment, the present invention relates to stable test samples that can be used in ligand binding assays, such as immunoassays. More specifically, the present invention relates

to stable test samples that contain at least one biological sample that contains at least one natriuretic peptide. The levels of the at least one natriuretic peptide in the biological sample can be quantified in the ligand binding assay. The biological sample is derived from plasma, serum, whole blood or other bodily fluids, such as, but not limited to, saliva, sputum, etc., as long as the 5 bodily fluids are stable in their collection technique. Additionally, the test samples of the present invention are stable for at least twenty-four (24) hours and can be used under a variety of storage conditions and temperatures.

As mentioned briefly above, the test sample contains at least one biological sample that is 10 derived from serum, plasma, whole blood or other bodily fluids and contains at least one natriuretic peptide. Preferably, the natriuretic peptide is a natural natriuretic peptide that is human and obtained from or contained within a biological sample derived from human serum, human plasma, human whole blood or other human bodily fluids. The human natriuretic peptide can be a human natural atrial natriuretic peptide, human natural B-type natriuretic peptide, 15 human natural C-type natriuretic peptide or human natural *Dendroaspis* natriuretic peptide.

In addition to the biological sample(s), the test sample contains a diluent that comprises a number of components. Any diluent can be used in the test sample of the present invention. For example, a diluent that can be used in the test sample of the present invention can be made using 20 techniques known in the art or can be purchased from a commercially available source. Combinations of both custom made diluents and commercially purchased diluents are also contemplated within the scope of the present invention.

The composition of the diluent can vary depending upon the test sample. For example, 25 the diluent can comprise water.

The diluent can also comprise at least one natriuretic stabilizing compound. As used herein, the term “natriuretic stabilizing compound” refers to a compound that can be used to stabilize a natriuretic peptide and prevent its degradation by enzymes, such as proteases. Several 30 natriuretic stabilizing compounds are known in the art and can be used in the calibrators and controls of the present invention. These compounds include, but are not limited to, proteins,

polymers and protease inhibitors. Examples of proteins that can be used include, but are not limited to, bovine serum albumin (BSA), bovine gamma globulin (bovine IgG), or non-fat dry milk (such as Nestlé® Carnation® Nonfat Dry Milk). Examples of polymers that can be used include, polyethylene glycol (having a molecular weight of from about 2,000 to about 20,000 daltons), dextran (having a molecular weight of from about 5,000 to about 670,000 daltons), dextran sulfate (having a molecular weight of about 5,000 daltons or about 1,000,000 daltons) or polyvinyl pyrrolidone (having a molecular weight of about 40,000 daltons). Bovine serum albumin, bovine IgG, polyethylene glycol, dextran, dextran sulfate and polyvinyl pyrrolidone can all be purchased from Sigma Aldrich, St. Louis, MO. Examples of protease inhibitors that can be used include, but are not limited to, EDTA or aprotinin. It is known in the art that EDTA at about 1mg/mL blood and aprotinin at approximately 50 kIU/mL of blood (Murdoch et al, *Heart*, 78:594-597 (1997)) can be used to help prevent BNP proteolysis. Protease inhibitors, such as EDTA and aprotinin can be purchased from Sigma Aldrich, St. Louis, MO. The amount of natriuretic stabilizing compound in the diluent can be from about 0.01 mg/mL to about 400 mg/mL, preferably in the amount of from about 1 mg/mL to about 50 mg/mL.

Additionally, the diluent can also comprise and at least one biocide and/or preservative. As used herein, the term “biocide” refers to a substance that can be used to kill a variety of different organisms. Suitable biocides and/or preservatives for use in the diluent can be determined using routine techniques by those skilled in the art. Examples of suitable biocides and/or preservatives that can be used in the present invention, include, but are not limited to, sodium azide and ProClin® 300 (Sigma-Aldrich, St. Louis, MO) (The active ingredients of ProClin® 300 are 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one. ProClin® 300 also contains a number of inert ingredients such as a modified glycol and alkyl carboxylate). The amount of biocide and/or preservative in the diluent can be from about 0.001 mg/mL to about 50 mg/mL, preferably in the amount of from about 0.1 mg/mL to about 10 mg/mL.

Furthermore, the diluent can also comprise at least one buffer, or at least one acid, or at least one base or combinations of at least one buffer, at least one acid and/or at least one base . Examples of buffers that can be used include, but are not limited to, acetate buffers (such as

sodium acetate/acetic acid, potassium acetate/acetic acid), citrate buffers (such as sodium citrate/citric acid, potassium citrate/citric acid), phosphate buffers (such as mono-/di-sodium phosphate) or combinations thereof. It is preferred that the buffer have a good buffering capacity at the desired pH so that it can stabilize the pH of the diluent and the resulting calibrator or
5 control. Examples of acids that can be used include, but are not limited to, acetic acid, diethylenetriaminepentaacetic acid (DTPA), hydrochloric acid (HCl) or combinations thereof. The amount of buffer or acid in the diluent can be from about 1 mM to about 500 mM, preferably in the amount of from about 5 to about 200 mM. An example of a base that can be used includes, but is not limited to, sodium hydroxide (NaOH). The amount of base in the
10 diluent can be from about 0.01 mM to about 50 mM, preferably in the amount of from about 1.0 mM to about 10 mM.

The diluent can also comprise sodium chloride (NaCl) and/or at least one detergent, such as Tween® (Any type of Tween® can be used, including, but not limited to, Tween® 20,
15 Tween® 40, Tween® 60, which are commercially available from Sigma-Aldrich, St. Louis, MO). The amount of NaCl in the diluent can be from about 25 mM to about 500 mM, preferably in the amount of from about 100 mM to about 400 mM. The amount of detergent in the diluent can be from about 0.01 mg/mL to about 10 mg/mL, preferably in the amount of from about 0.1 mg/mL to about 3.0 mg/mL.
20

In addition, as mentioned above, commercially available diluents can be used. For example, the diluent known as “MEIA2” that is commercially available as IMx MEIA #2 Diluent Buffer (No.8374-04) from Abbott Laboratories, the assignee of the present invention, can be used after adjustment of diluent buffer to desired pH as shown in Examples 1, 2 and 3
25 below. MEIA2 contains 0.06 M Tris buffer (Sigma-Aldrich, St. Louis, MO), 0.3 M NaCl, 0.1% w/v NaN₃ and has a pH of 7.5.

The pH of the diluent used in the test sample is from about 4.0 to about 6.0, preferably from about 5.4 to about 5.6. The amount of diluent contained in the test sample is from about
30 5% to about 95% (v/v) preferably, from about 20% to about 90% (v/v).

As mentioned above, the test samples of the present invention are stable. The key to the stability of the test sample of the present invention is pH. More specifically, it has been discovered that the test samples of the present invention are stable at a pH of from about 4.0 to about 6.5. Preferably, the pH of the test samples of the present invention is from about 5.0 to

5 about 6.0.

The test samples of the present invention having a pH of from about 4.0 to about 6.5 are stable for at least twenty-four (24) hours when used under laboratory conditions at ambient temperatures. In contrast, test samples known in the art are only stable for an hour or less after

10 they are diluted.

In another embodiment, the present invention relates to methods for making stable test samples for use in ligand binding assays, such as immunoassays. The method of the present invention involves mixing from about 5% to about 95% (v/v), preferably from about 20% to

15 about 90% (v/v) of at least one diluent having a pH of from about 4.0 to about 6.0, preferably from about 5.4 to about 5.6, with at least one biological sample derived from plasma, serum, whole blood or other bodily fluid and that contains at least one natriuretic peptide to form a stable, test sample. The diluent can have any of the compositions previously described herein and the natriuretic peptide can be any of the human natural natriuretic peptides also previously

20 described herein. Preferably, the diluent comprises at least one buffer having a good buffering capacity at the desired pH.

The diluent and biological sample can be mixed together at a temperature of from about 15° C to about 30°C, preferably at a temperature of from about 19°C to about 23°C, until a

25 homogenous solution is obtained. The pH of the resulting homogenous test sample can be in the range of from about 4.0 to about 6.5. After the stable test sample has been prepared, it can be used in an immunoassay according to standard protocols.

In another embodiment, the present invention relates to methods for stabilizing test samples for use in ligand binding assays, such as immunoassays. The method of the present invention involves mixing from about 5% to about 95% (v/v), preferably from about 20% to

about 90% (v/v) of at least one diluent having a pH of from about 4.0 to about 6.0, preferably from about 5.4 to about 5.6, with at least one biological sample derived from plasma, serum, whole blood or other bodily fluid and that contains at least one natriuretic peptide to form a stable, test sample. The diluent can have any of the compositions previously described herein
5 and the natriuretic peptide can be any of the human natural natriuretic peptides also previously described herein. Preferably, the diluent comprises at least one buffer having a good buffering capacity at the desired pH.

The diluent and biological sample can be mixed together at a temperature of from about
10 15° C to about 30°C, preferably at a temperature of from about 19°C to about 23°C, until a homogenous solution is obtained. The pH of the resulting homogenous test sample can be in the range of from about 4.0 to about 6.5. After the stabilized test sample has been prepared, it can be used in an immunoassay according to standard protocols. More specifically, the stabilized test
15 can be used immediately after it has been prepared in a ligand binding assay, or can be used at a much later period in time in a ligand binding assay. For example, when the stabilized test sample is maintained in a laboratory conditions at ambient temperatures, it can be used in a ligand binding assay at least about twenty-four (24) hours after being stabilized pursuant to the above-described method.

20 By way of example, and not of limitation, examples of the present invention will now be given.

EXAMPLE 1: BNP Immunoassay

Bulk diluents (“Dil”) having the following formulations were prepared:

25 Experimental Control Diluent = 4% BSA, 1% PEG, 0.1% Tween, 1% Dextran, 0.1% Proclin®
300, MEIA2 diluent.
Dil1 = 2% BSA, 0.1% Proclin® 300, MEIA2 diluent
Dil2 = Same as Dil1.
Dil3 = 2% BSA, 0.1% ProClin® 300, MEIA2 diluent and 0.1% Tween.
30 Dil4 = Same as Dil3.
Dil5= 2% BSA, 0.1% ProClin® 300, MEIA2 diluent and 10mM DTPA
Dil6 – Same as Dil5.
Dil7 = 2% BSA, 0.1% ProClin® 300, MEIA2 diluent, 0.1% Tween, 10mM DTPA.
Dil8 = Same as Dil7.

Dil9 = Same as Dil1.
Dil10 = Same as Dil2.
Dil11 = Same as Dil3.
Dil12 = Same as Dil4.
5 Dil13 = Same as Dil5.
Dil14 = Same as Dil6.
Dil15 = Same as Dil7.
Dil16 = Same as Dil8.

10 After each of the above diluents was prepared, the pH was determined. The pH of diluents 7-16 was adjusted. Specifically, for diluents that did not contain DTPA, HCl was added (drop wise) to adjust the pH (to about 5.6). For the diluents that contained DTPA, NaOH was used to adjust the pH (to about 5.6 or 7.4). The pH was not adjusted for diluents 1-4. The final pH for each diluent is shown below in Table 1.

15

Table 1

Dil1 – pH 7.41	Dil9 – pH 5.66
Dil2 – pH 7.41*	Dil10 – pH 5.66*
Dil3 – pH 7.39	Dil11 – pH 5.64
Dil4 – pH 7.39*	Dil12 – pH 5.64*
Dil5 – pH 7.41	Dil13 – pH 5.66
Dil6 – pH 7.41*	Dil14 – pH 5.66*
Dil7 – pH 7.40	Dil15 – pH 5.64
Dil8 – pH 7.40*	Dil16 – pH 5.64*

The pH of the experimental control diluent was 7.4.

* After final adjustment of the pH, these diluents (and the control) were also subjected to heat.

20 Specifically, these diluents were heated in a water bath at a temperature of about 56°C for about 1 hour.

Each of the above diluents was used to prepare three different calibrators containing different levels of human synthetic BNP (purchased from Peptide Institute (Osaka, Japan)).

25 Calibrator #A did not contain any human synthetic BNP. Calibrator #B contained 50 picograms/milliliter of human synthetic BNP. Calibrator #F contained 2000 picograms/milliliter of human synthetic BNP. Each calibrator was filtered and placed in calibrator bottles and stored at 37°C for up to 14 days.

A BNP immunoassay was performed on an AxSYM® instrument (this instrument is described in U.S. Patent No. 5,358,691). In addition to the calibrators prepared as described above, the following reagents were used in the assay:

5 1. Monoclonal antibody (MAb) 106.3 (capture antibody). This MAb binds to amino acids 5-13 on the BNP peptide. Monoclonal antibody 106.3 is commercially available from Scios, Inc. (Sunnyvale, CA) and is described in U.S. Patent No. 6,376,207. A microparticle was coated with MAb 106.3 using the techniques described in U.S. Patent No. 6,376,207, for use in the assay. Specifically, MAb 106.3 was coated onto 0.2 µm-size carboxylate modified

10 polystyrene particles (commercially available from Seradyn, Inc., Indianapolis, IN) using EDAC coupling (EDAC is generally used as a carboxyl activating agent for amide bonding with primary amines. In addition, it reacts with phosphate groups. It is used in peptide synthesis, crosslinking proteins to nucleic acids and in preparing immunoconjugates. The chemical formula for EDAC is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride. EDAC is commercially

15 available from Interchim (France)). Particles were washed and overcoated with BSA. After a final wash, particles were stored at 2-8°C in a buffer containing sucrose, sodium azide and BSA.

2. Monoclonal antibody BC203 (reporter antibody). This MAb binds to amino acids 27-32 on the BNP peptide. Monoclonal antibody BC203 is available from Shionogi, Inc. (Osaka, Japan). Monoclonal antibody BC203 is conjugated to alkaline phosphatase. BC203 was conjugated to alkaline phosphatase by Axis Shield Diagnostics (Dundee, Scotland, U.K.) in a typical coupling procedure using Traut's Reagent (which is 2-iminothiolane and is commercially available from Interchim (France)) and SMCC (SMCC is a heterobifunctional cross-linker whose formula is succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate. SMCC is commercially available from Interchim (France)). The stock conjugate was stored in a buffer containing BSA, fish gelatin, Brij-35 (Brij-35 is polyoxyethyleneglycol dodecyl ether and is a detergent that is commercially available from Sigma-Aldrich, St. Louis, MO) and sodium azide.

3. 4-methylumbelliferyl phosphate substrate (1.2 mM, in buffer, containing 0.1 sodium azide), which is commercially available from Abbott Laboratories, Abbott Park, Illinois.

4. The AxSYM® instrument system uses two (2) buffers: a Matrix Cell Wash and a Line Diluent. The Matrix Cell Wash is Tris buffered saline containing sodium azide and anti-microbial agents and is used to wash the particle reaction mixture. The Line Diluent is Phosphate buffer containing sodium azide and anti-microbial agents, and is used to rinse the 5 AxSYM® probes in between each aspiration. The Matrix Cell Wash and Line Diluent are commercially available from Abbott Laboratories.

The 0.2 μ m microparticles coated with the capture antibody in a buffer containing sucrose, sodium azide and BSA were pipetted by the sampling probe into the appropriate wells 10 of the reaction vessel in the sampling center. An aliquot containing one of the calibrators described above in Table 1 was delivered to the same well of the reaction vessel as the microparticles to form a reaction mixture. The reaction vessel was transferred to the processing center. The reaction mixture was incubated for approximately 12 minutes at a temperature of about 34°C. After the incubation, an aliquot of the reaction mixture was transferred to the matrix 15 cell of the AxSYM® instrument. The reaction mixture was then washed with the Matrix Cell Wash at 1 pulse of 100 μ L to remove any of the calibrator that was not captured.

MAb BC203-conjugate at about 0.75 μ g/mL in a buffer containing BSA, fish gelatin, Brij-3 and sodium azide, was dispensed onto the matrix cell and the resulting combination was 20 incubated for approximately 12 minutes at a temperature of about 34°C. The matrix cell was washed with the Matrix Cell Wash at 5 pulses of 50 μ L to remove the unbound materials.

A solution of the substrate, 4-methylumbelliferyl phosphate (MUP) in aminomethyl 25 phosphate buffer (1.2 mM) was added to the matrix cell and the rate of formation of 4-methylumbelliferone was measured by fluorescence reflectance. The fluorescent product, 4-methylumbelliferone, was measured by the microparticle enzyme immunoassay (MEIA) optical assembly of the AxSYM® instrument.

The AxSYM® system measured the alkaline phosphatase conversion of MUP to 4-methylumbelliferone (MU) by the rate of formation of the fluorescent product, MU. The 30 aforementioned rates are typically measured in counts per second per second. The assay was

conducted twice for each calibrator. The results in Table 2A below and Figure 1 show the mean of the two assays.

Table 2A
Calibrator stability at accelerated stability condition (37°C) for 0-14 days

Cals	0	2	5	7	9	12	14
Con-A	9.8	12.8	12.7	13.2	13.8	12.7	12.5
Con-B	39.9	41.6	37.9	39.4	37.8	33.8	30.9
Con-F	1725.2	1564.9	1410.1	1460.0	1477.6	1200.0	1107.9
1A	9.8	10.6	11.4	11.8	12.8	11.7	12.5
1B	37.3	33.6	30.6	30.2	32.9	29.2	26.4
1F	1703.2	1370.2	1184.4	1256.6	1276.4	1192.1	1102.4
2A	8.9	11.1	11.1	11.1	11.8	11.2	11.8
2B	41.6	39.4	36.2	37.4	38.1	35.8	31.9
2F	1865.9	1637.2	1398.4	1556.3	1562.7	1369.8	1309.2
3A	10.7	11.3	13.2	13.9	11.7	13.7	13.3
3B	34.4	39.9	33.6	37.9	36.0	34.0	31.7
3F	1746.1	1500.2	1325.2	1427.5	1315.0	1139.1	1199.9
4A	10.5	11.2	12.0	11.6	12.5	12.6	12.4
4B	32.8	39.6	33.9	36.2	36.9	32.5	32.7
4F	1611.8	1531.2	1236.9	1435.9	1418.2	1234.7	1190.8
5A	9.2	11.4	12.3	12.0	11.9	12.1	12.3
5B	37.4	38.7	34.8	40.1	38.1	36.1	32.3
5F	1671.1	1519.9	1285.2	1546.7	1444.3	1290.9	1246.3
6A	10.4	11.3	12.6	12.2	13.1	13.2	11.7
6B	38.7	38.1	37.1	38.1	39.0	33.9	32.1
6F	1721.3	1570.1	1350.8	1548.5	1455.7	1285.7	1387.7
7A	11.4	13.0	13.7	13.0	13.5	15.3	13.9
7B	44.8	47.3	41.5	45.5	47.0	39.0	38.3
7F	2036.9	1783.4	1531.5	1767.9	1787.4	1541.1	1483.2
8A	10.3	12.2	12.8	14.4	13.9	14.6	13.4
8B	38.5	43.5	35.4	39.4	39.8	35.6	35.1
8F	1637.5	1605.5	1305.2	1449.6	1468.7	1294.0	1286.0
9A	10.7	11.7	12.6	14.1	14.7	12.6	13.0
9B	43.8	55.9	47.8	50.1	54.1	49.0	45.2
9F	1807.9	2072.7	1707.4	1867.6	1998.2	1713.0	1634.9
10A	10.1	12.0	12.5	13.2	13.4	12.6	12.1
10B	48.7	55.8	49.7	51.9	59.2	48.5	46.5
10F	1794.0	2124.1	1928.3	1839.6	2142.1	1812.5	1648.3
11A	11.0	11.4	13.3	14.0	14.0	14.3	12.9
11B	39.0	49.0	42.8	43.5	49.2	43.4	42.2
11F	1606.1	1887.1	1593.5	1633.5	1836.5	1529.1	1637.9
12A	10.0	12.2	12.9	13.6	13.4	13.0	12.7
12B	44.4	58.0	46.4	49.4	53.7	48.4	48.6
12F	1784.8	1993.3	1759.2	1740.2	1839.2	1614.6	1629.7
13A	10.1	11.9	12.2	12.2	12.9	12.2	12.6
13B	49.2	64.1	58.2	58.7	62.1	54.0	54.4
13F	1996.7	2283.9	2023.6	2088.8	2283.5	1983.7	2171.4
14A	10.6	12.4	12.5	12.1	13.3	12.0	13.1
14B	42.7	56.4	54.0	56.5	56.8	47.8	52.6
14F	1864.3	2029.9	1861.4	1932.5	2064.8	1832.9	2092.6

15A	11.7	13.6	13.2	14.0	16.4	15.5	13.0
15B	49.8	62.7	54.7	58.2	59.1	56.5	55.4
15F	2106.2	2259.1	1995.2	2032.4	2247.6	2062.1	2150.2
16A	12.0	12.8	12.7	13.5	16.3	14.8	15.0
16B	44.8	57.0	50.3	52.4	60.5	48.4	54.1
16F	1801.6	1889.1	1889.4	1794.1	1977.2	1738.8	1930.6

Based upon the results shown about in Table 2A, the stability of calibrators 13, 14, 15 and 16 further examined at 21 days, 35 days, 49 days, 77 days, 96 days and 187 days. The results are shown below in Table 2B. The results shown are the mean of two assays.

5

TABLE 2B

	<u>21</u>	<u>35</u>	<u>49</u>	<u>77</u>	<u>96</u>	<u>110</u>	<u>125</u>	<u>131</u>	<u>159</u>	<u>187</u>
Con-A	13.7	14.8	14.9							
Con-B	27.5	26.6	24.1							
Con-F	992.2	795.4	650.6							
13A	13.4	13.8	13.4	14.8	20.6					
13B	46.8	48.6	50.0	45.4	46.6					
13F	1853.7	1895.4	1803.3	1579.1	1484.3					
14A	11.1	15.2	14.3	15.6	20.5					
14B	42.8	49.2	44.7	40.9	42.7					
14F	1712.0	1736.8	1694.1	1428.1	1291.5					
15A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	18.0
15B	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	42.6
15F	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1297.3
16A	12.8	16.0	15.1	16.6	20.9	#N/A	#N/A	#N/A	#N/A	18.7
16B	43.8	49.4	42.9	46.1	45.3	#N/A	#N/A	#N/A	#N/A	34.8
16F	1775.0	1646.2	1673.6	1490.2	1350.2	#N/A	#N/A	#N/A	#N/A	1030.6

10

The data in Table 2A and Figure 1 demonstrate that calibrators having a pH in the range of from about 4.0 to about 6.5, particularly at pH 5.6, exhibited enhanced accelerated stability compared to similar formulations with a pH of about 7.4. In calibrators 1-8 and the experimental control diluent calibrator, the rates for the "F" calibrators, i.e., 1F, 2F, 3F, 4F, 5F, etc. 15 (hereinafter referred to as the "Cal-F" rates) fell much faster through 14 days of stability study than the Cal-F rates in calibrators 9-16 which were similar formulations but at a different pH.

The enhanced accelerated stability can be quantified by comparing the average percentage change in F Calibrator rates at 37°C for 14 days for the eight F Calibrators at

approximately pH 7.4 (Calibrators 1F-8F; mean F Calibrator rate decreased by 27.1%) versus the eight F Calibrators in similar formulations but at approximately pH 5.6 (Calibrators 9F-16F; mean F Calibrator rate increased 0.9%). In each paired calibrator diluent (e.g., 1 and 9, 2 and 10, etc.) in which the formulation is identical except for pH, the F Calibrator rates dropped more for
5 those at approximately pH 7.4 than those at approximately pH 5.6. (Accelerated stability studies are well known in the art in which materials are stressed at a higher temperature than the intended storage condition. In describing a European standard for stability testing of *in vitro* diagnostic medical devices (EN 13640:2002), Armstrong describes incubation at an elevated temperature of 25°C, 37°C or 40°C as examples of accelerated stability testing. The Food and
10 Drug Administration also recognizes accelerated stability studies, for example, the Center for Drug Evaluation and Research, Manual of Policies and Procedures, issued directive MAPP 5226.1. Reaffirmation of expiration dating period for abbreviated applications. In the background section of this directive they describe accelerated stability conditions and data submittable for supporting stability (expiration dating) of generic drugs.)
15

EXAMPLE 2: BNP Immunoassay

Bulk diluents (“Dil”) of the following formulations were prepared.

Experimental Control Diluent = 4% BSA, 1% PEG, 0.1% Tween, 1% Dextran, 0.1% Proclin®
20 300, MEIA2 diluent.
Dil1 = 2% BSA, 0.1% Proclin® 300, MEIA2 diluent
Dil2 = Same as Dil1.
Dil3 = 2% BSA, 0.1% Proclin® 300, MEIA2 diluent and 0.1% Tween.
Dil4 = Same as Dil3.
25 Dil5 = 2% BSA, 0.1% Proclin® 300, MEIA2 diluent and 10 mM DTPA
Dil6 – Same as Dil5.
Dil7 = 2% BSA, 0.1% Proclin® 300, MEIA2 diluent, 0.1% Tween, 10mM DTPA.
Dil8 = Same as Dil7.
Dil9 = Same as Dil1.
30 Dil10 = Same as Dil2.
Dil11 = Same as Dil3.
Dil12 = Same as Dil4.
Dil13 = Same as Dil5.
Dil14 = Same as Dil6.
35 Dil15 = Same as Dil7.
Dil16 = Same as Dil8.

After each of the above diluents was prepared, the pH was determined. The pH of diluents 7-16 was adjusted. Specifically, for diluents that contained DTPA, NaOH was added (drop wise) to adjust the pH (to about 5.6). For the diluents that contained DTPA, NaOH was used to adjust the pH (to about 5.6 or 7.4). The pH was not adjusted for diluents 1-4. The final 5 pH for each diluent is shown below in Table 3.

Table 3

Dil1 – pH 7.41	Dil9 – pH 5.66
Dil2 – pH 7.41*	Dil10 – pH 5.66*
Dil3 – pH 7.39	Dil11 – pH 5.64
Dil4 – pH 7.39*	Dil12 – pH 5.64*
Dil5 – pH 7.41	Dil13 – pH 5.66
Dil6 – pH 7.41*	Dil14 – pH 5.66*
Dil7 – pH 7.40	Dil15 – pH 5.64
Dil8 – pH 7.40*	Dil16 – pH 5.64*

The pH of the experimental control diluent was 7.4.

10 * After final adjustment of the pH, these diluents (and the control) were also subjected to heat. Specifically, these diluents were heated in a water bath at a temperature of about 56°C for about 1 hour.

15 Each of the above diluents was used to prepare three different calibrators containing different levels of human synthetic BNP (purchased from Peptide Institute (Osaka, Japan). Calibrator #A did not contain any human synthetic BNP. Calibrator #B contained 50 picograms/milliliter of human synthetic BNP. Calibrator #F contained 2000 picograms/milliliter of human synthetic BNP. Each calibrator was filtered and placed in calibrator bottles and stored at 2-8°C for up to 271 days.

20 A BNP immunoassay was performed on an AxSYM® instrument as described in Example 1. The assay was conducted twice for each calibrator. The results in Table 4 below and Figure 2 show the mean of two assays.

Table 4
 Calibrator stability at 2-8°C for up to 271 days

<u>Cals</u>	<u>0</u>	<u>2</u>	<u>5</u>	<u>7</u>	<u>9</u>	<u>12</u>	<u>14</u>	<u>243</u>	<u>271</u>
Con-A	9.8	13.2	12.3	14.6	14.8	14.8	14.4	19.9	16.4
Con-B	39.9	52.7	48.3	54.3	57.9	51.0	58.3	43.8	39.9
Con-F	1725.2	1988.8	1867.6	1949.6	2229.4	1901.8	2123.8	1199.9	1239.2
1A	9.8	12.7	11.5	12.8	12.9	11.9	11.8	20.7	15.3
1B	37.3	49.4	45.3	50.2	51.4	46.5	52.8	39.0	36.4
1F	1703.2	1928.9	1887.0	2004.1	2183.3	1846.8	2196.8	1125.8	1181.3
2A	8.9	11.6	11.7	12.3	12.6	11.5	11.5		
2B	41.6	52.4	50.0	56.6	63.8	53.8	57.7		
2F	1865.9	2255.0	2055.9	2170.1	2298.1	2186.9	2327.8		
3A	10.7	12.1	12.4	13.0	14.6	15.6	14.4	21.7	17.2
3B	34.4	47.1	43.9	52.3	49.6	48.3	51.5	39.6	36.8
3F	1746.1	1940.5	1919.3	2103.5	2173.1	1879.1	2232.9	1021.9	1030.5
4A	10.5	11.8	12.5	13.7	14.8	15.3	14.0	#N/A	16.8
4B	32.8	42.6	42.8	45.4	47.1	44.9	48.6	#N/A	39.6
4F	1611.8	1863.5	1792.4	1859.7	1973.7	1781.1	2026.9	#N/A	1190.4
5A	9.2	12.0	12.1	13.9	12.3	12.0	12.2	21.1	15.1
5B	37.4	46.7	43.6	48.8	55.5	48.8	50.9	44.0	39.9
5F	1671.1	1989.7	1907.4	1976.4	2133.1	1910.4	2110.1	1230.4	1319.5
6A	10.4	12.8	11.6	13.3	12.7	12.5	13.7		
6B	38.7	44.9	43.8	46.3	50.0	45.2	50.9		
6F	1721.3	1710.0	1906.6	1845.7	1954.2	1791.3	2027.4		
7A	11.4	13.3	15.2	16.4	14.1	15.0	14.6	24.1	19.2
7B	44.8	52.7	60.2	58.5	63.7	56.5	65.4	48.0	41.5
7F	2036.9	2120.2	2150.2	2205.2	2340.9	2138.6	2405.4	1329.9	1386.0
8A	10.3	13.0	12.9	15.1	14.2	16.0	14.6		
8B	38.5	43.2	46.6	49.6	51.9	49.0	55.3		
8F	1637.5	1700.3	1765.6	1728.0	1856.6	1703.4	1963.0		
9A	10.7	13.5	12.9	13.5	12.6	11.6	12.3	21.5	16.3
9B	43.8	51.5	56.6	54.4	60.1	56.5	61.9	55.7	50.4
9F	1807.9	1855.8	1985.5	2042.5	2078.3	2012.2	2382.1	1586.5	1669.1
10A	10.1	11.8	12.1	12.3	12.9	12.1	11.3		
10B	48.7	50.4	50.4	54.7	56.6	56.0	65.1		
10F	1794.0	1922.5	2119.9	2051.4	2153.9	2130.1	2387.8		
11A	11.0	12.5	12.1	13.4	14.9	14.5	14.6	22.5	16.9
11B	39.0	42.9	46.7	51.3	53.9	46.4	54.0	50.2	45.8
11F	1606.1	1606.4	1864.1	1942.5	1894.7	1740.9	1992.9	1361.7	1444.0
12A	10.0	12.3	13.0	14.9	14.6	14.3	14.5		
12B	44.4	49.4	57.5	62.8	58.5	54.4	62.9		
12F	1784.8	1844.2	1999.4	2206.1	2074.8	2072.6	2389.0		
13A	10.1	11.8	12.8	11.1	12.0	12.4	11.0		
13B	49.2	53.9	62.3	69.2	62.3	61.3	65.1		
13F	1996.7	2037.5	2187.6	2382.9	2225.7	2148.3	2168.7		
14A	10.6	11.5	13.1	13.8	12.3	12.9	12.5		
14B	42.7	48.6	56.0	62.2	54.0	51.7	51.6		

14F	1864.3	1820.8	2135.9	2194.2	2090.4	2093.0	2000.9		
15A	11.7	13.8	14.4	13.8	15.5	14.2	15.3	22.2	17.7
15B	49.8	54.5	63.3	68.2	61.8	61.9	57.2	55.9	53.2
15F	2106.2	2059.9	2354.6	2414.5	2191.5	2130.9	2209.0	1722.8	1805.3
16A	12.0	12.2	14.0	15.2	14.7	14.3	14.2	21.8	DEPLETED
16B	44.8	48.0	53.0	59.9	55.1	53.3	54.5	50.5	DEPLETED
16F	1801.6	1760.3	1943.0	2093.6	1907.9	1866.5	1861.8	#N/A	DEPLETED

The data in Table 4 and Figure 2 demonstrate that calibrators having a pH in the range c from about 4.0 to about 6.5 (calibrators 9-16) exhibited less of a signal decrease than the
5 corresponding calibrators 1-8 at a pH of about 7.4. This enhanced long term stability was observed through 271 days at 2-8°C.

EXAMPLE 3: BNP Immunoassay

Bulk diluents ("Dil") of the following formulations were prepared.

Dil1 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA
5 Dil2 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
Dil3 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
Dil4 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
Dil5 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
10 Dil6 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
Dil7 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
Dil8 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
Dil9 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
Dil10 = 2% BSA, 0.1% Proclin® 300, 10 mM DTPA, 100 mM NaCl, 0.1% sodium azide in water
15

In addition, to the components listed above, diluent 1 also contained MEIA2 as a base diluent. Diluents 3-6 also contained a solution of sodium acetate and a solution of acetic acid. Diluents 7-10 also contained a solution of monobasic sodium phosphate and a solution of dibasic sodium phosphate.

20

After each of the above diluents was prepared, the pH was determined. The pH of diluents 3-10 was adjusted. Specifically, the pH of diluents 3-10 was adjusted with NaOH. The pH was not adjusted for diluents 1-2. The final pH for each diluent is shown below in Table 5.

25

Table 5

Dil1 – pH 5.66*	Dil6 – pH 5.49*
Dil2 – pH 3.91	Dil7 – pH 6.10*
Dil3 – pH 3.47	Dil8 – pH 6.51*
Dil4 – pH 4.50	Dil9 – pH 7.04*
Dil5 – pH 5.02	Dil10 – pH 10.04*

* After final adjustment of the pH, these diluents were also subjected to heat. Specifically, these diluents were heated in a water bath at a temperature of about 60°C for about 1 hour.

30

Each of the above diluents was used to prepare three different calibrators containing different levels of human synthetic BNP (purchased from Peptide Institute (Osaka, Japan)). Calibrator #A did not contain any human synthetic BNP. Calibrator #B contained 50 picograms/milliliter of human synthetic BNP. Calibrator #F contained 2000 picograms/milliliter

of human synthetic BNP. Each calibrator was filtered and placed in calibrator bottles and stored at 37°C and 2-8°C for up to 252 days.

A BNP immunoassay was performed on an AxSYM® instrument as described in
 5 Example 1, the only change being that about 0.50 µg/mL of MAb BC203-conjugate in a conjugate buffer containing BSA, fish gelatin, Brij-35 and sodium azide was used. The assay was conducted twice for each calibrator. The results in Table 6 below and Figure 3 show the mean of the two assays.

10

TABLE 6
Calibrator stability at 37°C or 2-8°C for up to 252 days

<u>37°C</u>	<u>0</u>	<u>3</u>	<u>7</u>	<u>14</u>	<u>21</u>	<u>28</u>	<u>35</u>	<u>56</u>	<u>77</u>	<u>91</u>	<u>106</u>	<u>112</u>	<u>140</u>	<u>168</u>	<u>193</u>	<u>224</u>	<u>252</u>
1A	14.0	12.3	12.8	12.4	11.5	13.8	14.5	18.9	16.7	19.0	#N/A	25.3	31.8	36.5			
1B	92.0	74.5	76.8	79.9	61.9	68.7	62.9	64.1	49.6	50.6	#N/A	47.7	51.4	60.2			
1F	2413.72318.62056.32200.72004.31943.71803.71524.11499.21248.8#N/A										1065.4	784.6	726.2				
5A	11.5	12.7	14.4	17.9	11.4	16.0	15.2	17.6	17.2	17.5	#N/A	23.1	28.5	38.8			
5B	96.0	81.5	77.5	76.6	61.8	70.6	67.0	61.6	58.1	55.4	#N/A	53.7	58.7	73.9			
5F	2600.22406.22206.82212.02053.62078.92001.71784.21800.41712.5#N/A										1598.9	1375.2	1512.3				
2A	11.9	28.0	104.2	49.7													
2B	91.7	68.2	95.4	58.5													
2F	2477.42080.154.7		122.3														
4A	11.3	35.6	27.7	55.8	#N/A	#N/A	#N/A	#N/A	57.1								
4B	87.1	74.6	82.4	77.2	#N/A	#N/A	#N/A	#N/A	53.2								
4F	1966.61769.21686.91901.0#N/A				#N/A	#N/A	#N/A	#N/A	85.0								
6A	12.6	15.6	14.5	13.8	12.3	15.0	15.0	18.2	16.1	17.9	#N/A	26.4	28.6	44.3	31.3		
6B	86.3	117.7	149.2	67.4	53.7	61.3	59.2	53.8	43.9	45.9	#N/A	49.1	48.1	58.1	41.8		
6F	2548.62359.52187.12134.91997.11902.51857.11653.41553.21517.0#N/A										1266.3	1167.2	1061.1885.9				
7A	11.4	11.2	17.3	12.5	11.6	14.8	13.5	17.3	18.4	17.2	#N/A	26.5	27.5	40.7	48.4		
7B	99.0	71.3	70.5	68.4	56.5	55.6	50.5	46.8	40.5	37.7	#N/A	44.9	42.6	62.8	48.4		
7F	2617.02308.42056.52120.11887.11780.31641.61326.01157.21117.5#N/A										901.0	759.3	744.5	619.0			
8A	11.4	11.3	17.6	16.2	10.3	12.6	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	31.3		
8B	92.1	65.9	60.5	55.9	44.0	43.7	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	37.8		
8F	2474.92073.81882.41754.61572.61385.9#N/A						#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	362.5		
9A	12.5	10.4	13.9	14.3	13.3	13.8											
9B	96.3	63.3	58.4	47.4	36.7	34.6											
9F	2686.12175.31940.81643.51495.31158.3																
3A	11.3	40.6	29.2	42.1													
3B	25.7	24.9	20.7	30.7													
3F	533.9	28.9	23.4	27.8													
10A	15.1	12.4	15.4	13.9													
10B	46.4	21.7	18.4	13.5													
10F	1518.8663.9	261.9	83.4														

<u>2-8°C0</u>	<u>3</u>	<u>7</u>	<u>14</u>	<u>21</u>	<u>28</u>	<u>35</u>	<u>56</u>	<u>77</u>	<u>91</u>	<u>106</u>	<u>112</u>	<u>140</u>	<u>168</u>	<u>193</u>	<u>224</u>	<u>252</u>	
1A	14.0	11.3	17.8	15.7	12.9	13.4	13.4	16.9	16.6	17.3	17.2	27.6	#N/A	#N/A	depleted		
1B	92.0	83.0	100.0	89.8	79.0	80.7	76.6	77.7	71.5	77.9	65.3	79.3	84.8	#N/A	depleted		
1F	2413.72435.92330.22333.42429.02396.12269.72086.22068.81971.22024.21828.91799.31940.6														depleted		
5A	11.5	10.6	13.9	12.2	11.4	14.0	13.1	18.5	19.2	17.9	18.3	22.2	28.3	44.5	depleted		
5B	96.0	86.3	88.4	92.5	79.1	88.5	81.5	75.6	70.0	67.6	67.4	77.2	79.7	#N/A	depleted		
5F	2600.22480.72279.12548.52518.72415.92379.82210.62056.52157.92217.01992.11872.0#N/A														depleted		
2A	11.9	10.7	17.4	14.5	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	19.4	16.4	
2B	91.7	80.6	87.0	78.0	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	47.0	46.3	
2F	2477.42420.92341.02430.7#N/A				#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1586.41577.9		
4A	11.3	10.2	14.6	15.0	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	28.0	16.8	17.8
4B	87.1	75.7	85.1	84.1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	79.3	56.7	61.1
4F	1966.61926.31788.12098.1#N/A				#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1501.0	1380.21373.2	
6A	12.6	11.2	15.6	17.3	11.5	14.4	13.7	17.7	17.2	19.7	#N/A	29.0	29.8	42.4	31.5	21.9	19.5
6B	86.3	69.2	78.2	80.1	72.6	73.3	66.1	68.4	56.9	60.1	#N/A	65.8	70.6	81.0	77.1	54.0	50.3
6F	2548.62423.72307.82469.52529.92405.92236.62105.02032.91966.8#N/A											1871.11754.61816.01634.6			1608.21591.8		
7A	11.4	10.7	13.1	12.1	11.8	16.5	15.6	18.2	16.3	18.5	#N/A	27.7	31.1	42.1	32.3	20.5	18.4
7B	99.0	84.7	89.3	89.6	87.0	87.6	83.8	82.9	73.0	70.5	#N/A	76.3	74.7	97.4	79.1	52.8	51.9
7F	2617.02511.72478.82634.62619.52504.02370.42184.62157.22016.7#N/A											1898.11788.81940.91809.8			1579.41653.1		
8A	11.4	11.0	14.2	12.7	12.5	14.2	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	31.4	18.0	16.5
8B	92.1	84.7	84.7	86.7	73.4	75.8	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	64.2	45.4	43.9
8F	2474.92524.42286.12489.02354.12284.6#N/A						#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1617.1	1430.41389.9	
9A	12.5	10.3	14.0	13.0	12.5	15.1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	22.4	19.1
9B	96.3	87.1	95.5	91.2	84.0	86.3	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	42.3	42.7
9F	2686.12682.72508.22662.32448.32372.0#N/A						#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1478.51497.4	
3A	11.3	9.8	16.8	13.6													
3B	25.7	28.6	33.9	29.6													
3F	533.9	560.8	602.5	545.7													
10A	15.1	11.9	17.5	16.6													
10B	46.4	37.3	41.2	37.4													
10F	1518.81407.21226.01238.1																

The data in Table 6 and Figure 3 demonstrate that calibrators having a pH in the range of from about 4.0 to about 6.5 (calibrators in diluents 1, 4-8) exhibited less of a signal decrease than the calibrators in diluents 2-3 (at pH's of 3.91 and 3.47, note that #3 had low signal even at 0 time) and calibrators in diluents 9-10 (pH's 7.04 and 10.04) at 37°C accelerated stability. At 2-8°C, little change in F Cal rates was observed through 14 days except for diluent #3 which had low signal even at 0 time and for diluent # 10 which decreased by 18.5%.

10 EXAMPLE 4: STABILIZED TEST SAMPLE

Three (3) human plasma samples with levels of BNP greater than 2000 pg/mL were obtained from patients with heart failure. 120 µL of each specimen was diluted with 480 µL of AxSYM® BNP Standard Calibrator A (0.32 M acetate buffer, 2% BSA (w/v), 10 mM DTPA, 0.09% sodium azide (w/v), 0.1 ProClin® 300 (w/v), pH 5.5) (“Calibrator A”). Another 120 µL of each specimen was obtained and was not diluted with Calibrator A (these samples are referred to as “undiluted plasma samples”). Immediately after dilution, the undiluted plasma samples (referred to herein as “neat”) and the diluted plasma samples were placed on the AxSYM® instrument system and analyzed for BNP using the AxSYM® BNP reagent kit (The AxSYM® BNP reagent kit contains the AxSYM® BNP Reagent Pack (the AxSYM® BNP Reagent Pack contains 1 bottle (8.4 mL) Anti-BNP (Mouse Monoclonal) coated microparticles in Tris Buffer, 1 bottle (13.2 mL) Anti-BNP (Mouse, Monoclonal) alkaline phosphatase conjugate in TRIS buffer with protein stabilizers, and 1 bottle (14.1 mL) wash buffer containing detergent), Reaction Vessels and Matrix Cells). The neat sample underwent an auto-dilution (1/5) by the AxSYM® instrument and the diluted samples were tested directly. Following analysis, the remaining neat samples and diluted samples were stored at room temperature (22°C) for four (4) hours. After this time period, all of the diluted samples were reanalyzed in the same manner as they were originally tested. The results from this testing are shown below in Table 7. The results demonstrate that the samples diluted with Calibrator A (described above) remain stable (97% recovery) following four (4)

hour storage at room temperature, whereas the neat samples, stored under these same conditions, showed only 85% recovery.

TABLE 7

5

sample	0 time			4 hrs Room Temp.			
	Auto dilution	Manual dilution in Cal A	% 0 time Auto dilution	Auto dilution	% 0 time Auto dilution	Manual dilution in Cal A	% 0 time Auto dilution
6BNP04	2908	3033	104%	2676	92%	3015	104%
BNP15	2673	2493	93%	2432	91%	2541	95%
BNP27	3349	3083	92%	2452	73%	3119	93%
Average recovery=			97%		85%		97%

EXAMPLE 5: STABILIZED DILUTED SAMPLE

Nine (9) human plasma samples with levels of BNP greater than 400 pg/mL,

10 including three of the samples from Example 4, were obtained from patients with heart failure. 120 µL of each specimen was diluted with 480 µL of AxSYM® BNP Standard Calibrator A (0.32 M acetate buffer, 2% BSA (w/v), 10 mM DTPA, 0.09% sodium azide (w/v), 0.1 ProClin® 300 (w/v), pH 5.5) (“Calibrator A”). Another 120 µL of each specimen was obtained and was not diluted with Calibrator A (these samples are referred 15 to as “undiluted plasma samples”). Immediately after dilution, all of the undiluted plasma samples (referred to herein as “neat”) and the diluted plasma samples were placed on the AxSYM® instrument system and analyzed for BNP using the AxSYM® BNP reagent kit (The AxSYM® BNP reagent kit contains the AxSYM® BNP Reagent Pack (the AxSYM® BNP Reagent Pack contains 1 bottle (8.4 mL) Anti-BNP (Mouse 20 Monoclonal) coated microparticles in Tris Buffer, 1 bottle (13.2 mL) Anti-BNP (Mouse, Monoclonal) alkaline phosphatase conjugate in TRIS buffer with protein stabilizers, and 1 bottle (14.1 mL) wash buffer containing detergent), Reaction Vessels and Matrix Cells). Following analysis, all of the diluted samples were stored at room temperature (22°C). After four (4), five (5) and twenty-four (24) hours storage, the samples were 25 reanalyzed in the same manner as they were originally tested. The results from this

testing are shown below in Table 8. The diluted concentrations were multiplied by their dilution factors (5) and then divided by the corresponding neat values to calculate dilution recovery. The results demonstrate that the samples diluted with Calibrator A (described above) remained stable following 4, 5 or 24 hour (102%, 97%, and 111%
5 dilution recovery respectively) storage at room temperature.

TABLE 8

Sample	Neat Value	Diluted concentrations				Dilution Recovery			
		0 time	4 hours	5 hours	24 hours	0 time	4 hours	5 hours	24 hours
A	2908	607	603	-	-	104%	104%	-	-
B	2673	499	508	-	-	93%	95%	-	-
C	3349	617	624	-	-	92%	93%	-	-
D	1500	302	-	302	-	101%	-	101%	-
E	2219	547	-	517	-	123%	-	116%	-
F	1680	300	-	327	-	89%	-	97%	-
G	883	206	-	204	215	117%	-	116%	122%
H	2160	456	-	479	541	106%	-	111%	125%
I	825	160	-	162	161	97%	-	98%	98%
J	494	96	-	101	100	97%	-	102%	101%
		average=		102%	97%	106%	111%		

10 EXAMPLE 6: COMPARISON WITH OTHER SAMPLE DILUENTS

Four (4) human plasma samples with BNP levels greater than 180 pg/mL, were obtained from patients with heart failure. 120 µL of each specimen was diluted with 480 µL of AxSYM® BNP Standard Calibrator A (0.32 M acetate buffer, 2% BSA (w/v), 10 mM DTPA, 0.09% sodium azide (w/v), 0.1 ProClin® 300 (w/v), pH 5.5) (“Calibrator
15 A”). Another 120 µL of each specimen was diluted with 480 µL of Multi-diluent I (Bayer Healthcare LLC, Tarrytown, NY). Another 120 µL of each specimen was obtained and was not diluted with Calibrator A or Multi-diluent I (these samples are referred to as “undiluted plasma samples”). Immediately after dilution, all of the undiluted plasma samples (referred to herein as “neat”) and all of the diluted plasma
20 samples (both those diluted with Calibrator A and with Multi-diluent I) were placed on the AxSYM® instrument system and analyzed for BNP using the AxSYM® BNP reagent kit (The AxSYM® BNP reagent kit contains the AxSYM® BNP Reagent Pack (the AxSYM® BNP Reagent Pack contains 1 bottle (8.4 mL) Anti-BNP (Mouse Monoclonal) coated microparticles in Tris Buffer, 1 bottle (13.2 mL) Anti-BNP (Mouse,

Monoclonal) alkaline phosphatase conjugate in TRIS buffer with protein stabilizers, and 1 bottle (14.1 mL) wash buffer containing detergent), Reaction Vessels and Matrix Cells). Following analysis, all of the diluted samples were stored at room temperature (22°C). After five (5) hours and twenty-four (24) hours storage, the samples were 5 reanalyzed in the same manner as they were originally tested. The results from this testing are shown below in Table 9. The diluted concentrations were multiplied by their dilution factors (5) and then divided by the corresponding neat values to calculate dilution recovery. The results demonstrate that the samples diluted with Calibrator A (described above) remain stable following five (5) or twenty-four (24) hours (106% and 10 112% recovery respectively) storage at room temperature with a dilution recovery of 110% of expected (range from 102% to 120%). The samples diluted in Multi-diluent I showed progressive decline over time with recoveries at 5 and 24 hours of 67% and 54%. The 0 time dilution recovery was 121% (range from 84% to 216%).

15

Table 9

Sample	Neat Value	Diluted concentrations			Dilution Recovery		
		0 time	5 hours	24 hours	0 time	5 hours	24 hours
Standard Calibrator A diluent							
A	2015	438	408	413	109%	101%	102%
B	2069	448	455	505	108%	110%	122%
C	1005	205	208	-	102%	103%	-
D	183	44	40	-	120%	109%	-
Multi-diluent (Bayer)							
A	2015	359	280	201	89%	69%	50%
B	2069	400	338	238	97%	82%	58%
C	1005	168	137	-	84%	68%	-
D	183	79	18	-	216%	49%	-

Calibrator A average= 110% 106% 112%
 Multi-diluent average= 121% 67% 54%

All abstracts, references, patents and published patent applications referred to herein are hereby incorporated by reference.

5

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof.

10 Changes can be made to the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention.

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